



Antioxidant activity and enzyme inhibition of phenolic acids from fermented rice bran with fungus *Rhizopus oryzae*



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ABSTRACT

The solid-state fermentation (SSF) has been employed as a form making available a higher content of functional compounds from agroindustrial wastes. In this work, the effect of SSF with the *Rhizopus oryzae* fungus on the phenolic acid content of rice bran was studied. Phenolic extracts derived from rice bran and fermented rice bran were evaluated for their ability to reduce free radical 1,1-diphenyl-2-picrihydrazil (DPPH) and for the ability to inhibit the enzymes peroxidase and polyphenol oxidase. The phenolic compound content increased by more than two times with fermentation. A change in the content of phenolic acids was observed, with ferulic acid presenting the greatest increase with the fermentation, starting from 33 mg/g in rice bran and reaching 765 mg/g in the fermented bran. The phenolic extracts showed an inhibition potential for DPPH and for the peroxidase enzyme, however did not inhibit the polyphenol oxidase enzyme.

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1. Introduction

Fermentation processes have been studied for many decades. Solid state fermentation (SSF) is a simple technique for the production of bioactive compounds. It is economically viable due to the use of agro-industrial residues, and also helps reduce the environmental impact of their disposal (Oliveira et al., 2010; Schmidt & Furlong, 2012).

One of the most produced and consumed grains in the world, rice (*Oryza sativa*) is a rich source of bioactive compounds, including many phenolic antioxidants (Mira, Barros, Schiocchet, Noldin, & Lanfer-Marquez, 2008; Zhang, Zhang, Zhang, & Liu, 2010). These have the potential to reduce the risk of disease and can be applied in the food industry, as well as in the cosmetics and health markets (Butsat & Siriamornpun, 2010; Pourali, Asghari, & Yoshida, 2010). Phenols are an important class of chemical compounds which can be divided into two subgroups according to their structure, *p*-hydroxybenzoic acid derivatives such as gallic, protocatechuic and syringic acids and hydroxycinnamic derivatives such as caffeic, ferulic, *p*-coumaric and chlorogenic acids (Martins et al., 2011).

One of the main byproducts of rice processing is bran. Rice bran has 11–13% protein, approximately 11% fiber and 20% of its weight in oil, as well as containing functional compounds and antioxidants (Oliveira et al., 2011). Traditionally, most rice bran production was

used in the production of fertilizers, animal feed and the cosmetic industry, but several studies have been conducted to better assess its potential for human consumption (Silveira & Furlong, 2007).

A number of processes have been developed in order to increase the synthesis of biologically active microbial metabolites (Membriño, Sánchez, Meneses, Favela, & Loera, 2011). SSF is a way of providing a higher content of phenolic compounds from agro-industrial residues (Martins et al., 2011). Phenolic compounds are found in plants as defense mechanisms and with other biological functions, including metal chelation, the sequestering of some active oxygen species and antioxidant activity (Nara, Miyoshi, Honma, & Koga, 2006).

Among the major microorganisms known for their ability to produce enzymes that degrade the cell wall of plants, fungi comprise the most interesting group (Hegde, Kavitha, Varadaraj, & Muralikrishna, 2006). The genus *Rhizopus* is one of the most promising in this process because it has been shown that, besides the ability to increase the protein content of the raw materials of low nutritional value, these proteins possess functional activity and specific catalytic activity. Furthermore, the fungi of this genus are well indicated for not producing toxic substances (Oliveira et al., 2010).

The aim of this study was to determine the profile of phenolic acids derived from solid state fermentation of rice bran with the fungus *Rhizopus oryzae* and evaluate the antioxidant capacity and inhibition of enzymes peroxidase and polyphenol oxidase by extracts containing these compounds.

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2. Material and methods

2.1. Fermentation of the rice bran

2.1.1. Preparation of inoculum

The fungus *R. oryzae* (CCT 1217), was obtained from the André Tosello Foundation (FAT), Campinas, Brazil. The cultures were maintained at 4 °C in slants of potato dextrose agar (PDA, Acumedia®). The spores were spread by adding 5 mL of an aqueous emulsion (Tween 80 at 0.2%v/v) and they were incubated for 7 days at 30 °C until a whole new sporulation of the fungus by adding 0.2 mL of the emulsion in Petri dishes containing potato dextrose agar. Spore suspension for fermentation was achieved by adding 10 mL of an aqueous emulsion of Tween 80 (0.2%) to each plate. The release of spores was obtained by scraping the plates with a Drigalski handle and the concentrated spores solution was estimated by enumeration in a Neubauer chamber (L. Opatik, Germany).

2.1.2. Fermentation process

The rice bran (rice variety BR-IRGA 417) used as substrate in fermentation was provided by industries from Rio Grande do Sul, with their particles size standardised to particles smaller than 32 mesh, and packed in 100 g in tray bioreactors (12 × 8 × 4 cm³) arranged in 2 cm layers, covered with sterilized gauze and cotton to allow aeration and prevent external contamination. The reactors containing the substrate were added in a nutrient solution (2 g/L KH₂PO₄, 1 g/L MgSO₄ and 8 g/L (NH₄)₂SO₄ in 0.4 N HCl) sterilized by filtration in Millipore membrane of 0.45 µm (Oliveira et al., 2010).

The spores solution of the fungus *R. oryzae* was added at an initial concentration of 4 × 10⁶ spores/g_{bran}. Distilled water was added to the medium in order to adjust the humidity to 50%. The bioreactors were placed in a fermentation chamber at 30 °C with controlled humidity. Upon expiry of the incubation time (0–120 h, with sampling every 24 h), the fermented biomass was stored at –18 °C.

2.1.3. Biomass

The biomass generated during the fermentation process was indirectly estimated by the glucosamine content (Aidoo, Henry, & Wood, 1981). The glucosamine content was estimated spectrophotometrically (Biospectro, Brazil) at 530 nm using a standard curve of glucosamine (Sigma, USA) in water (1–15 mg/mL). The amount of glucosamine in rice bran (not fermented) was subtracted from the fermented bran with the biomass being expressed as mg_{glucosamine}/g_{bran} in dry base.

2.2. Extraction of phenolic compounds

Phenolic compounds from fermented rice bran were extracted with methanol at a ratio of 1:10 (w/v), following the method described by Souza, Oliveira, Rocha, and Furlong (2010). Samples of 5 g were subjected to orbital shaking (150 rpm) at room temperature for 3 h with methanol and the extract obtained was filtered through filter paper (Whatman n° 4) into a separating funnel and washed three times with 10 mL of hexane. The methanolic extract was evaporated on a rota-evaporator at 50 °C under reduced pressure and the phenolic compounds were resuspended with 10 mL of distilled water in an ultrasonic bath for 10 min. The resulting extract was clarified with 5 mL of 0.1 M ZnSO₄ and 5 mL of 0.1 M Ba(OH)₂, and allowed to rest for 20 min. After centrifugation (10 min, 25 °C, 3200g,) the supernatant containing the phenolic compound was collected, lyophilized and quantified spectrophotometrically at 750 nm with Folin–Ciocalteu reagent (Qell, Brazil) using ferulic acid (Sigma, Japan) as standard (2–20 µg/mL).

2.3. Separation and identification of phenolic acids

Phenolic extracts were resuspended in water and methanol (1:1), and 20 µL aliquots injected into a chromatograph (Shimadzu, Tokyo, Japan, CLASS-M10A) at a flow rate of 0.7 mL/min at 35 °C. The separation of the phenolic acids was accomplished using a C18 column (4.6 × 250 mm, 5 µm, Discovery®, USA) and a gradient isocratic solvent consisting of methanol and acidified water (1% v/v acetic acid) at a 20:80 ratio during 25 min, with UV detection at 280 nm until 15 min and 320 nm until 25 min. Phenolic acids were identified by comparison of retention times and absorption spectrum with different standards of phenols present in rice bran (caffeic, chlorogenic, *p*-coumaric, ferulic, gallic, *p*-hydroxybenzoic, protocatechuic, syringic and vanillin, obtained from Sigma–Aldrich, USA) as described in the literature (Mira et al., 2008; Pourali et al., 2010). The detection limit (LOD) was calculated by the background noise signal (solution containing the solvents used in the extraction of phenolic compounds) at 3:1. The determination limit (LOQ) was established as three times the amount of the LOD (Ribani, Bottoli, Collins, Jardim, & Melo, 2004).

2.4. Antioxidant activity of phenolic extracts

The phenolic antioxidant activity of the extracts was determined according to the methods described by Rufino, Fernandes, Alves, and Brito (2009), Sánchez-Moreno, Larrauri, and Saura-Calixto (1998) and Brand-Williams, Cuvelier, & Berset, 1995 measured by the reduction in free radical 1,1-diphenyl-2-picrihydrazil (DPPH). This method is based on the transfer of electrons from one antioxidant substance to a free radical, DPPH, which loses its purple colour upon reduction, becoming yellow. Different concentrations of solutions of ascorbic acid (0.01–0.1 mg/mL), ferulic acid (0.01–1 mg/mL), fermented and unfermented rice bran (0.01–0.5 mg/mL) were tested, so that it could get different values of DPPH at steady state covering the widest range between 0 and 100%. In the dark, 0.2 mL of the sample was added to 3.8 mL of 0.5 mM DPPH. The consumption of DPPH was monitored by spectrophotometer at 515 nm for different reaction times, until its stabilization. The DPPH concentration in the medium was calculated using a calibration curve (0–0.16 mg/mL) and determined by linear regression (Eq. (1)).

$$A_{515\text{nm}} = 6.6953 \times [\text{DPPH}] \quad (r = 0.999) \quad (1)$$

where: [DPPH] = concentration of DPPH expressed in mg/mL.

From the calibration curve equation, the percentage of the remaining DPPH for each time at every concentration tested was determined according to Eq. (2):

$$\% \text{DPPH}_{\text{REM}} = ([\text{DPPH}]_t / [\text{DPPH}]_{\text{control}}) \times 100 \quad (2)$$

The DPPH_{REM} percentage was plotted against the reaction time using an exponential model of the first order, through the software *Microcal Origin 6.0*, to estimate the percentage of DPPH_{REM} at steady state for each concentration tested. And then the percentage of DPPH_{REM} at steady state was plotted against the solutions concentration to obtain the amount of antioxidant needed to decrease the initial concentration of DPPH by 50% (EC₅₀). The time needed to reach the EC₅₀ (T_{EC50}) was obtained graphically as proposed by Sánchez-Moreno et al. (1998). The anti-radical efficiency (AE) was calculated according to Eq. (3).

$$\text{AE} = 1 / (\text{EC}_{50} * T_{\text{EC50}}) \quad (3)$$

2.5. Enzymatic inhibition of phenolic extracts

The inhibitory effect of phenolic compounds produced by the fermentation was evaluated on the enzymes responsible for browning in plant tissues, peroxidase and polyphenol oxidase.

The enzyme extract was obtained from 20 g of potato (*Solanum tuberosum* L., Monalisa variety) with 100 ml of buffer solution pH 7 (0.1 M phosphate-citrate buffer). After 2 min of grinding in a blender, the mixture was filtered (by cotton) and centrifuged (15 min, 4 °C, 3200g). The crude enzyme extract was used as the enzyme source, with the soluble protein content estimated in mg of albumin (Lowry, Rosenbrough, Farr, & Randall, 1951). The peroxidase enzyme activity was determined using 0.2 ml of enzyme extract, 1 ml of 30 mM H₂O₂, 2 mL of a 5 mM guaiacol solution, with the final volume of the tube being completed to 4 ml with buffer pH 7, and the reaction absorbance detected at 470 nm after 10 min of reaction at 30 °C. The polyphenol oxidase activity was determined using 1 ml of enzyme extract, 2 mL of a solution of 10 mM catechol, 1 mL of buffer pH 7 with the absorbance reaction detected at 425 nm after 10 min of reaction at 30 °C. The inhibitory effect of phenolic compounds extracted from rice bran and fermented rice bran (96 h) in the activity of these enzymes was evaluated using different concentrations of the inhibitor. The final pH of the reaction was adjusted at 7 by the addition of a solution of 0.1 M NaOH.

The inhibition mechanism of phenolic compounds on the peroxidase enzyme was also evaluated by the k_m and V_{max} parameters. Different concentrations of the substrate (guaiacol) were used in the enzymatic reaction with the addition of phenolic extract solutions. The results were plotted according to Lineweaver & Burk (1934) graphic method.

2.6. Statistical analysis

One-way Analysis of Variance (ANOVA) test was used to determine significant differences between variables. Differences with a probability value of <0.05 were considered significant and all data were reported as mean \pm sd.

3. Results and discussion

3.1. Biomass and phenolic content

After fermentation time of 48 h, there was not detected a significant increase in phenolic content, whereas the fungal biomass demonstrated an important increased until 96 h of fermentation (Fig. 1). The glucosamine, a constituent of chitin, an insoluble linear polymer composed of α -1,4 acetylglucosamine bonds, was determined to estimate the multiplication in fungal SSF (Schmidt & Furlong, 2012). At 96 h, 8.8 mg_{glucosamine}/g were obtained from fermented biomass, showing that the *R. oryzae* fungus can grow using rice bran as a carbon source.

The phenolic compounds content at the beginning of fermentation was of about 2.4 mg/g and at the end of 120 h was of 5.1 mg/g, resulting in an increase of over 110% (Fig. 1). Rice phenolics include derivatives of benzoic and hydroxycinnamic acids, mainly ferulic acid and diferulates. These are commonly present in a chain form, and are normally components of complex structures such as hydrolyzable tannins and lignins, and linked to the cell wall structural components such as cellulose, lignin and proteins by ester linkages (Zhang et al., 2010). The more soluble phenolics are compartmentalised within the cell vacuoles, and they are in free or conjugated form, while the insoluble phenolics are connected to structures in the cell walls, esterified with arabinose or galactose residues of hemicellulose or pectic components (Mira, Massaretto, Pascual, & Marquez, 2009; Mira et al., 2008).

There are two ways in which phenolic compounds can be formed; from the decomposition of the linkages between lignin, cellulose and hemicellulose or by producing a part of rice bran oil (Pourali et al., 2010). In the case of rice bran fermentation, the

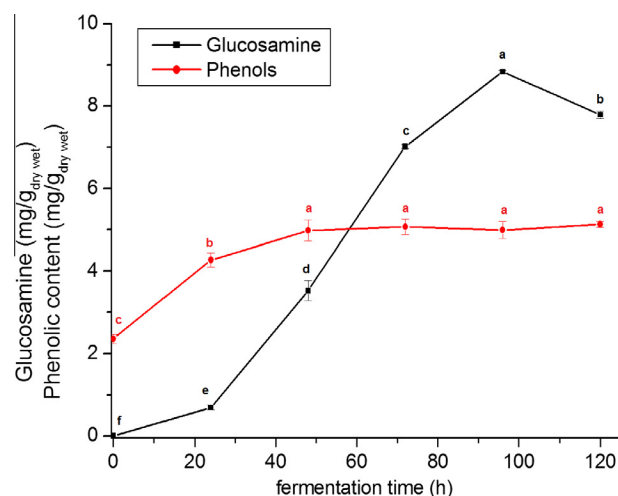


Fig. 1. Glucosamine and phenolic content during fermentation of rice bran. The point values in each line with the same superscript letter are not significantly different by Tukey test ($p < 0.05$).

increased phenolic acids content is mainly caused by the cleavage of compounds complexed with lignin (Schmidt & Furlong, 2012). Filamentous fungi produce a range of enzymes required to break the lignin, and these microorganisms have two extracellular systems, one that produces carbohydrases and another ligninolytic oxidative system which degrades phenyl rings, increasing the free phenolic content (Martins et al., 2011; Sánchez, 2009).

3.2. Phenolic acids of fermented rice bran

Supplementary data 1 and 2 show the calibration parameters and the separation of the group of phenolic acids that were analysed using an isocratic gradient elution. One can observe that the content of rice bran phenolic acids varied with the autoclaving treatment (time zero) but the major change in the content of these compounds occurred with fermentation (Table 1).

Among phenolic compounds the *p*-coumaric acid was the only one that did not display a significant increase ($p < 0.05$) in its content at the end of fermentation; it showed increased zero time (due to the heat treatment) that remained after 24 h, reduced content after 72 h, possibly due to oxidation processes during fermentation, and a small increase ($p < 0.05$) up to 120 h, which still failed to exceed the content of unfermented rice bran (RB). Ryan et al. (2011) also noted a reduced *p*-coumaric acid content after fermenting rice bran with *S. boulardii*.

Chlorogenic and *p*-hydroxybenzoic acids and vanillin showed an increase in their content throughout the fermentation. Protocatechuic acid did not show any significant increase ($p < 0.05$) after 24 h, whereas gallic and caffeic increased until 72 h, and syringic and ferulic acids increased their content until up to 120 h of fermentation. Gallic and ferulic acid contents displayed the most substantial content increases during fermentation, of about 60 and 20 times, respectively, compared to their contents in unfermented rice bran. The changes produced by fermentation on the profile of phenolic acids depend on the type of substrate, the fungus used and the conditions of fermentation (Martins et al., 2011; Schmidt & Furlong, 2012).

Agro-industrial residues of vegetables and cereals such as bran, bagasse, straw, corn cob, among others are lignocellulosic materials mainly composed by cellulose, hemicellulose and lignin. The lignin fraction of these materials contains numerous phenolic compounds, mainly acids such as ferulic, coumaric, syringic and hydroxybenzoic, which can also be recovered by SSF. Since fungi grow on these residues, they use the polysaccharides after lignin

Table 1
Phenolic acid content during of the fermentation (mg/g_{dry wet}).

Phenolic acid	RB	Fermentation time (h)					
		0	24	48	72	96	120
Gallic	2.6 ± 0.8 ^c	nd	3.6 ± 0.3 ^c	76.4 ± 5.6 ^b	170.2 ± 24.2 ^a	166.9 ± 6.9 ^a	154.5 ± 6.0 ^a
Protocatechuic	7.7 ± 1.4 ^c	8.7 ± 1.2 ^{bc}	12.5 ± 1.9 ^{ab}	8.4 ± 0.7 ^{bc}	12.2 ± 1.8 ^{ab}	10.2 ± 2.7 ^{abc}	13.6 ± 0.4 ^a
Chlorogenic	20.9 ± 0.7 ^d	14.6 ± 0.5 ^d	4.8 ± 1.2 ^d	126.5 ± 12.6 ^a	137.4 ± 0.8 ^a	100.6 ± 10.4 ^b	76.1 ± 1.7 ^c
p-Hydroxybenzoic	2.4 ± 0.4 ^c	6.2 ± 1.8 ^c	19.1 ± 1.9 ^b	30.3 ± 2.1 ^a	23.6 ± 0.3 ^b	29.2 ± 3.1 ^a	22.3 ± 2.6 ^b
Caffeic	4.8 ± 0.9 ^{bc}	1.6 ± 0.2 ^c	2.4 ± 0.2 ^c	11.2 ± 0.4 ^b	27.7 ± 1.5 ^a	27.0 ± 4.9 ^a	28.7 ± 5.1 ^a
Syringic	2.1 ± 0.3 ^d	2.6 ± 0.6 ^d	7.6 ± 1.6 ^c	9.2 ± 0.1 ^{bc}	6.9 ± 0.1 ^c	10.8 ± 1.9 ^{ab}	12.7 ± 0.5 ^a
Vanillin	8.6 ± 0.4 ^d	20.3 ± 1.4 ^a	14.1 ± 0.1 ^b	22.0 ± 0.5 ^a	14.1 ± 0.4 ^b	10.3 ± 1.3 ^{cd}	13.1 ± 1.9 ^{bc}
p-Coumaric	14.9 ± 0.9 ^b	41.3 ± 4.9 ^a	40.5 ± 2.7 ^a	2.5 ± 0.6 ^c	5.5 ± 0.8 ^c	12.7 ± 1.1 ^b	14.1 ± 1.1 ^b
Ferulic	33.3 ± 2.3 ^{de}	16.8 ± 3.8 ^e	10.5 ± 2.8 ^e	68.4 ± 6.1 ^d	386.8 ± 2.4 ^c	602.6 ± 16.2 ^b	764.7 ± 32.0 ^a

RB = rice bran, nd = not detected. Values are expressed as means ± sd. The values in each column with the same superscript letter are not significantly different by Tukey test ($p < 0.05$).

degradation in order to grow and reproduce (Martins et al., 2011; Sánchez, 2009).

Ferulic acid was the phenolic compound that stood out during the fermentation process, with over 700 mg/g produced (Table 1). The release of ferulic acid from agricultural byproducts by enzymatic methods has been increasingly researched, with most studies using yeast as an enzyme source (Martins et al., 2011). Ferulic acid has commercial potential, and may be applied as a natural precursor of vanillin, natural antioxidant, preservative agent in food products, anti-inflammatory agent and photo-shield (Yang, Yue, Cao, Zhang, & Wang, 2009). Vanillin is one of the most commonly used flavouring agents in food products, fragrances, beverages and pharmaceuticals, and has recently been indicated in the bioconversion of ferulic acid in order to decrease vanillin production costs (Zheng et al., 2007). Our results suggest that the use of the *R. oryzae* fungus in rice bran could produce the enzymes capable of releasing ferulic acid residues and agro-industrial byproducts.

3.3. Antioxidant activity

The antioxidant activity of the phenolic compounds was evaluated by inhibiting free radical DPPH, expressed in terms of the ability to reduce/sequester the free radical. Compared to others, this is a widely used method to evaluate the antioxidant capacity in a short time interval (Rufino et al., 2009; Sánchez-Moreno et al., 1998).

Ascorbic acid is one of the most potent antioxidant compounds used in food formulations (Pineli & Moretti, 2007) whereas ferulic acid was the main phenolic compounds found in fermented and unfermented rice bran (Schmidt & Furlong, 2012). The inhibitive power of ascorbic acid was above 95% of radical at a concentration of 0.1 mg/ml, whereas at the same concentration, the other extracts failed to inhibit 50% of the free radical (Fig. 2). Ascorbic acid reached steady state in less than 1 min (Fig. 2a), whereas the ferulic acid solution reached steady state in a shorter time (Fig. 2b) than solutions of rice bran (Fig. 2c) and fermented bran extracts (Fig. 2d), thus indicating that the mixture of phenolics in these extracts slowed down inhibition.

The concentration of antioxidant required to reduce the initial concentration of DPPH by 50% (EC_{50}) is the most commonly used parameter to measure the antioxidant properties of a substance (Rufino et al., 2009); the lower the EC_{50} value, the higher its antioxidant power. Although the phenolic extract of fermented rice bran presented a lower antioxidant power (Table 3), it showed an EC_{50} value close to the values of ferulic acid and unfermented rice bran solutions. The EC_{50} values of these extracts were lower than the values found for cardamom and onion extracts (Mariutti, Barreto, Bragagnolo, & Mercadante, 2008) and white rice bran obtained from different cultivars (Muntana & Prasong, 2010).

The ascorbic acid solution showed an EC_{50} value about 2.5 times lower than the other antioxidant solutions. But the EC_{50} value does not take into consideration the time to reach steady state of the inhibition reaction. According to the kinetic classification based on the time needed to reach the EC_{50} value (Sánchez-Moreno et al., 1998; Brand-Williams et al., 1995), ascorbic acid exhibited a fast antioxidant action, whereas ferulic acid and rice bran (fermented and unfermented) solutions displayed intermediate and slow actions, respectively (Table 2).

Another kinetic classification of antioxidant solutions which takes into account the concentration and EC_{50} time, called antiradical efficiency (AE), indicates that while the ascorbic acid solution demonstrated very fast AE, the other solutions exhibited a low AE (Table 2), and the fermented and unfermented rice bran solutions displayed lower efficiency than the ferulic acid solution, caused by the presence of other phenolic compounds of slow AE contained in these extracts. The lower AE of fermented rice bran extract compared to rice bran can be compensated by increasing phenolic content in the fermentation (Fig. 1).

The efficiency of phenolic compounds as antioxidants depends largely on their chemical structures, relative orientation and number of hydroxyl groups attached to the aromatic ring (Sánchez-Moreno et al., 1998). The importance of evaluating the reaction rate is related to how these extracts may be added as an antioxidant to protect certain food products for a long period of storage, or during processing, for which stability is needed in the conditions employed. For example, a slow-acting antioxidant must be added to frozen-stored products and a quick-acting antioxidant should be used in baked or fried products (Mariutti et al., 2008).

Although the phenolic extract of fermented rice bran has shown a small loss of antioxidant activity with respect to the phenolic extract of unfermented rice bran, in terms of EC_{50} and AE, the high increase in phenolic content with fermentation offsets this loss.

Phenolic compounds derived from rice bran fermentation with the *R. oryzae* fungus display antioxidant activity. The production and extraction of bioactive compounds through fermentation is an alternative that deserves attention, since it provides high quality extracts and biological activity with little or no toxicity usually associated with the organic solvents used for the extraction of these compounds (Martins et al., 2011, Nigam, 2009).

3.4. Enzyme inhibition

Enzymatic browning is an undesirable reaction that occurs in fruits and vegetables. The browning reaction requires the presence of oxygen, phenolic compounds and oxidative enzymes (Pineli & Moretti, 2007). Thus, antioxidant compounds with similar potential to those in this study are used to inhibit enzymatic browning.

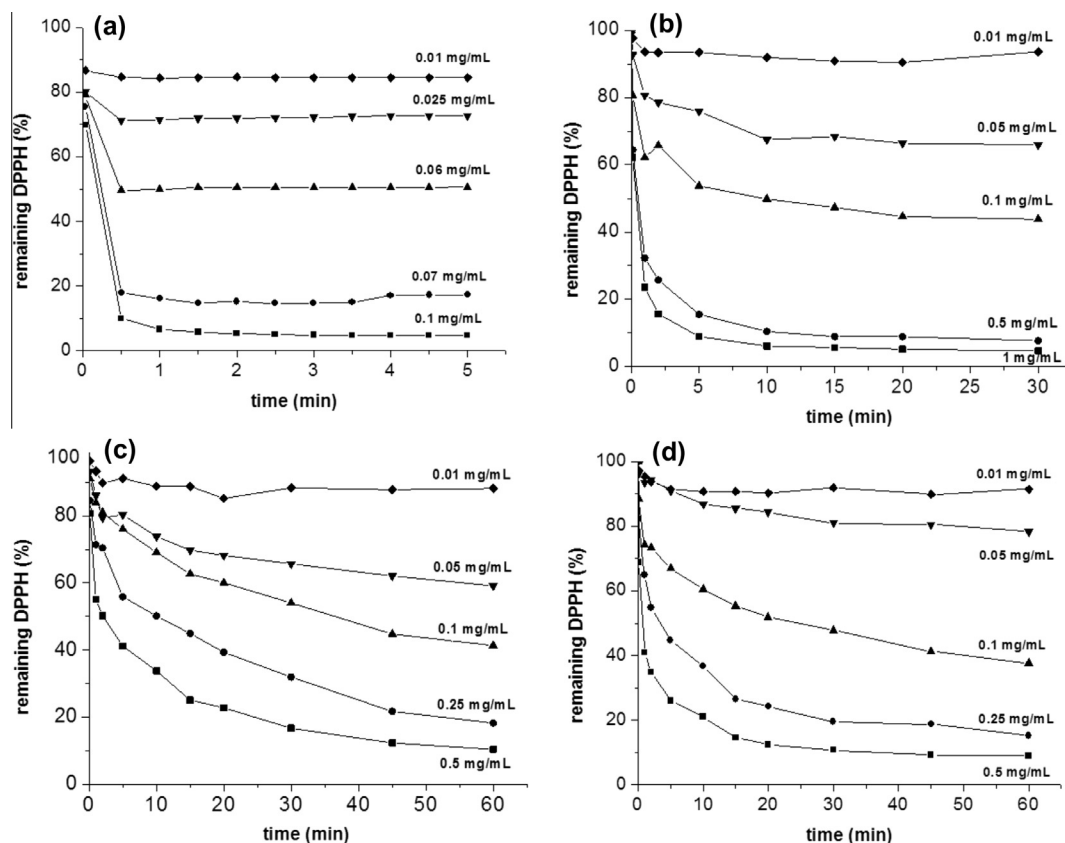


Fig. 2. Kinetic behaviour of the solutions antioxidants (a) ascorbic acid, (b) ferulic acid, (c) rice bran and (d) fermented rice bran.

Table 2

Values of EC_{50} and antiradical efficiency of the solutions antioxidants.

Antioxidant solution	EC_{50} (mg _{antiox.} /g _{DPPH})	EC_{50} time (min)	Classification	AE ($\times 10^3$)	Classification
Ascorbic acid	98 ± 16^d	0.37 ± 0.03^d	Rapid	27.30	Very high
Ferulic acid	235 ± 4^b	20.74 ± 0.74^c	Intermediate	0.21	Low
Rice bran	213 ± 10^c	40.53 ± 0.96^b	Slow	0.12	Low
Fermented rice bran	250 ± 4^a	43.05 ± 0.49^a	Slow	0.09	Low

AE = antiradical efficiency. Values are expressed as means \pm sd. The values in each line with the same superscript letter are not significantly different by Tukey test ($p < 0.05$).

Table 3

Values of km e V_{max} for peroxidase enzyme using inhibitor extracts of rice bran and rice bran fermented.

Reaction	[Inhibitor] mg/mL	$km \times 10^3$ (mM)	V_{max} (1/min)	km/V_{max} (mM/min)	Inhibitor type
Substrate + S1	0	1.19	0.167	7.13	Uncompetitive
	0.05	0.50	0.060	8.33	
	0.15	0.32	0.037	8.65	
Substrate + S2	0	0.65	0.101	6.44	Competitive
	0.05	0.34	0.087	3.91	
	0.15	0.17	0.092	1.82	
Substrate + S3	0	0.65	0.078	8.39	Competitive
	0.05	0.26	0.091	2.85	
	0.15	0.06	0.068	0.89	

S1 = standard solution of ferulic acid; S2 = phenolic extract solution from rice bran; S3 = phenolic extract solution from fermented rice bran; km = Michaelis–Menten constant.

Bearing that in mind, phenolic extracts of the control rice bran and fermented rice bran were evaluated for their ability to inhibit polyphenol oxidase and peroxidase enzymes. The antioxidant solutions showed greater inhibition of the peroxidase enzyme, with the solutions of ferulic acid and from fermented and unfermented rice bran showing a similar inhibitory power, reaching close to 60%

inhibition when was used a concentration of about three times the value of their EC_{50} (approximately 0.1 mg/ml) was used (Fig. 3).

The polyphenol oxidase was not inhibited at any concentration of antioxidant solutions from fermented and unfermented (control) rice bran extracts, while the solution of ferulic acid showed greater inhibition power at a concentration corresponding to three

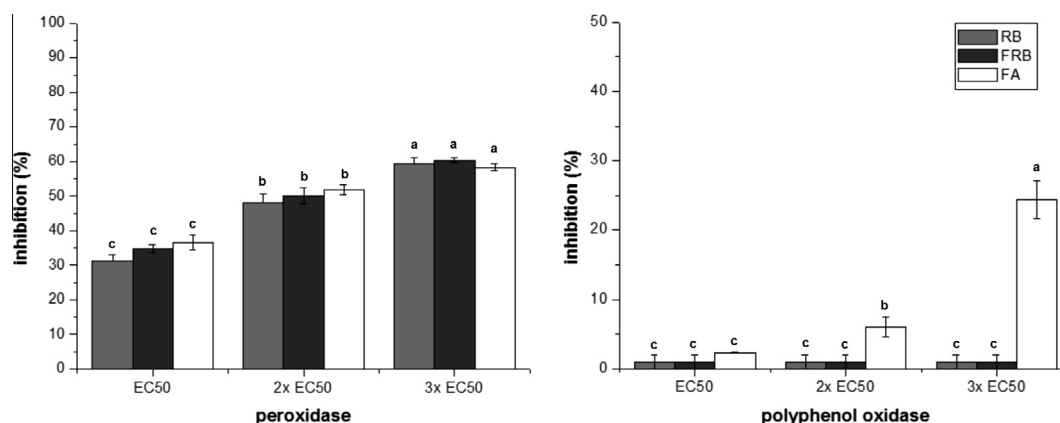


Fig. 3. Enzymatic inhibition by antioxidants solutions of rice bran (RB), fermented rice bran (FRB), ferulic acid (FA) in different concentrations corresponding EC₅₀ value. The values in each bar with the same superscript letter are not significantly different by Tukey test ($p < 0.05$).

times the EC₅₀. The fact that the phenolic extracts are not effective inhibitors of the polyphenol oxidase enzyme, even with high ferulic acid content, shows that the extracts have phenolic compounds which also serve as substrate for this enzyme, as in the case of chlorogenic, caffeic and gallic acids (Queiroz, Silva, Lopes, Fialho, & Valente-Mesquita, 2011).

The polyphenol oxidase catalyses the oxidation of polyphenols to quinones which react non-enzymatically to produce coloured pigments whereas peroxidase is capable of oxidising phenolic compounds in the presence of hydrogen peroxide (Pineli & Moretti, 2007; Queiroz et al., 2011). The potato enzyme extract showed greater peroxidase enzyme activity (0.24 AU/min*mg_{protein}) than for polyphenol oxidase (0.06 AU/min*mg_{protein}), which behaviour has also been observed by other authors (Cantos, Tudela, Gil, & Espín, 2002; Pineli, Moretti, Almeida, Onuki, & Nascimento, 2005), and the polyphenol oxidase enzyme can release H₂O₂, thus increasing peroxidase enzyme activity (Pineli & Moretti, 2007).

The standard solution of ferulic acid showed an uncompetitive inhibition (Supplementary data 3A), where the value of k_m and V_{max} decreased with the inhibitor addition, but the k_m/V_{max} ratio hardly changed (Table 3). Such behaviour differed from that of the solutions of fermented and unfermented rice bran, which displayed similar inhibitory behaviour (Supplementary data 3B and C); where the k_m values decreased and V_{max} values showed little change with the inhibitor addition (Table 3). This behaviour indicates a competitive inhibition (Whitaker, 1994), and therefore the phenolic compounds are similar to the preferred enzyme substrate. Although these solutions presented a greater ferulic acid concentration, especially in the fermented extract solution, the results show that the phenolic acids mixture influence the peroxidase enzyme inhibition, indicating that phenolic acids present in the extracts compete with substrate molecules for the active centre of the enzyme.

SSF has been used to increase the content of phenolic compounds in certain food products, thus enhancing their antioxidant activity. Accordingly, different agro-industrial residues have been used as solid substrates in SSF for the production of different bioactive phenolic compounds (Martins et al., 2011). The results of this study show that fermentation led to an increased free phenolic compound content in the rice bran, which has an antioxidant activity potential to inhibit free radical and peroxidase enzyme action. They can also be applied to products aimed the inhibiting this enzyme, as fruit juices or in development of minimally processed vegetable products (Rico, Martín-Diana, Barat, & Barry-Ryan, 2007; Singh et al., 2010). Furthermore, these compounds can be used for conversion into other compounds of interest, such as ferulic acid into vanillin.

4. Conclusion

Solid state fermentation of rice bran with the *R. oryzae* fungus increased free phenolic content by more than 100%. A change in the profile of the phenolic acids was observed, with gallic and ferulic acids presenting the highest increase with the fermentation, reaching 170 and 765 mg/g, respectively. The phenolic extract from fermented rice bran showed slow inhibition kinetics of the DPPH radical, presenting an EC₅₀ value of 250 mg/g_{DPPH} and potential competitive-type inhibition for the peroxidase enzyme.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2013.09.101>.

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